xGen™ cfDNA & FFPE DNA Library Prep Kit for the Singular Genomics G4™ Sequencing Platform

The method presented here is provided by IDT. This method uses the xGen™ cfDNA & FFPE DNA Library Prep MC v2 Kit with Singular Genomics Dual Index PCR Primers and can be used as a starting point for creating next generation sequencing (NGS) libraries intended for downstream sequencing on the G4 Platform. This method can be used in similar experiments but may not be fully optimized for your application. IDT does not guarantee these methods, and application scientists at IDT can only provide general guidance with limited troubleshooting support. For more information and support for Singular Genomics products and sequencing on the G4 Platform, see Singular Genomics website.

Overview

The xGen cfDNA & FFPE DNA Library Prep v2 MC Kit is designed specifically for 1–250 ng of degraded samples, such as cell-free DNA (cfDNA) or DNA extracted from formalin-fixed, paraffin-embedded (FFPE) samples. The technology utilized for this library construction workflow enables high conversion of degraded or damaged samples into sequencer-ready libraries without the need for adapter titration. The kit also allows for independent tagging of the top and bottom strands of the library, using our proprietary ligation strategy with fixed UMI sequences. This demonstrated protocol describes the workflow for preparing libraries for whole genome sequencing (WGS) on the G4 Sequencing Platform, including optional hybridization capture following library prep. For sequencing, the Singular Dual Index PCR Primers must be used with the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit to create compatible libraries for the G4 Sequencing Platform.

There are four major steps to perform in this protocol:

- **End repair.** Converts cfDNA, or sheared input DNA, into blunt-ended DNA ready for ligation.
- **Ligation 1.** Ligation 1 Enzyme catalyzes the single-stranded addition of the Ligation 1 Adapter to the 3′ ends of the insert DNA.
- **Ligation 2.** Ligation 2 Adapter acts as a primer to gap-fill the bases complementary to the UMI, followed by ligation to the 5′ end of the DNA insert to create a fully double-stranded product.
- **PCR amplification.** xGen 2x HiFi PCR Mix with Singular Dual Index PCR Primers are used to perform indexing PCR for sequencing on the G4 Sequencing Platform.

1	End repair		Total time: 35 min
2	End repair cleanup	2.5X AMPure	Total time: 30 min
3	Ligation 1		Total time: 40 min
	⏸		
4	Ligation 2		Total time: 45 min
5	Ligation 2 cleanup	2.5X PEG/NaCl	Total time: 45 min
	⛔		
6	PCR amplification using Singular index primers		Total time: 15–30 min (Variable depending on input)
7	PCR cleanup	1.3X AMPure	Total time: 40 min
	⛔		
8	Optional hybridization capture		

Consumables and equipment

Consumables—IDT

Item	Catalog #
xGen cfDNA & FFPE DNA Library Prep MC v2 Kit	10010207
IDTE pH 8.0 (1X TE Solution), 300 mL	11-05-01-13

Consumables—Other suppliers

Item	Supplier	Catalog#
Dual Index PCR Primers (1-96)	Singular Genomics	700,110
Buffer EB (10 mM Tris-HCl, pH 8.5), 250 mL	Qiagen	19086
Absolute ethanol (200 proof)	Various	Varies
Purification beads		
Agencourt® AMPure® XP-PCR Purification Beads 5 mL (22 rxn) or 60 mL (270 rxn)	Beckman Coulter	A63880 or A63881
Digital electrophoresis chips and associated reagents (choose one):		
Experion® DNA 1K Analysis Kit	Bio-Rad	700-7107
High Sensitivity DNA Kit	Agilent	5067-4626
High Sensitivity D1000 ScreenTape®	Agilent	5067-5584
twin.tec® 96 Well LoBind® PCR Plates	Eppendorf	0030129504
DNA LoBind® Tubes, 1.5 mL	Eppendorf	022431021
Filtered low bind pipette tips	Various	Varies
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific	032851 or 032854

Equipment

Item	Supplier	Catalog#
Microcentrifuge	Various	Varies
Thermal cycler	Various	Varies
Qubit 4 Fluorometer, or similar DNA quality analyzer	Thermo Fisher Scientific	033226
Magnet options (choose one):		
Permagen 96-well side pull magnet	TMO	NC1568572
Magnum® EX Universal Magnet Plate	Alpaqua	A000380
Magnetic Stand-96, or similar magnetic stand	Thermo Fisher Scientific	AM10027

Guidelines

Reagent storage and handling

Always store the xGen cfDNA & FFPE DNA Library Prep v2 MC Kit reagents at –20°C, except for PEG/ NaCl, which can be stored either at room temperature, or at –20°C.

Note: The enzymes provided in this kit are temperature-sensitive and appropriate care should be taken during storage and handling.

Note: The xGen 2x HiFi PCR Mix may be in a liquid state after storage in –20°C condition; this is expected and will not impact product integrity.

For all non-enzyme reagents, thaw on ice, then briefly vortex to mix well. Remove enzyme tubes from –20°C storage and place on ice just before use. Briefly centrifuge the tubes in a microcentrifuge to collect contents before opening.

To create master mixes, scale reagent volumes as appropriate, using 10% excess volume to compensate for pipetting loss. Add reagents in the order listed when preparing master mixes, then pulse-vortex to mix and briefly centrifuge.

Note: You may observe precipitation in the End Repair Buffer. Continue vortexing until no precipitate can be observed.

Important: Use extra caution when handling Ligation 1 and Ligation 2 adapter tubes. Never handle Ligation 2 adapter prior to, or during Ligation 1 Master Mix setup and handling. Trace contamination of Ligation 2 adapter into Ligation 1 adapter has been shown to induce adapter-dimer formation.

DNA input considerations

This kit works with a wide range of DNA inputs, ranging from 1–250 ng. Input DNA should be stored in IDTE buffer (10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA) or Buffer EB (Qiagen, 10 mM Tris HCl, pH 8.5).

Important: Input quantities recommended in this protocol refer to the total DNA quantified after fragmentation.

DNA should be an appropriate size before library construction. For genomic DNA, or DNA derived from FFPE samples, using a Covaris® or similar DNA shearing instrument can create fragments with an average insert size of 150–300 base pairs. As cfDNA typically has an average size of 160 base pairs, no further fragmentation is required.

For FFPE samples, use standard quality control methods, such as Q-ratio with qPCR or the DNA Integrity Number (DIN) using size distribution (ie., Bioanalyzer instrument (Agilent) or similar DNA quality analyzer).

These methods can help you choose the appropriate number of PCR cycles for your DNA sample. For cfDNA, we suggest assessing the size distribution with electrophoresis. Note, if large molecular weight DNA is present, an additional cleanup may be necessary to remove genomic DNA contamination. However, this additional cleanup can reduce sample complexity and mass.

Note: For more information on avoiding cross contamination, and size selection during clean-ups see the full xGen cfDNA & FFPE DNA Library Prep v2 protocol.

Protocol

End repair

Before starting the protocol, ensure that the AMPure and PEG/NaCl reagents are at room temperature (20–25°C). Also, prepare a fresh 80% ethanol solution in nuclease-free water.

1. Add 50 µL of each sample into a low-bind PCR plate that resists nucleic acid adsorption.

   Note: If sample volume <50 µL, use IDTE buffer (10 mM Tris-Cl, pH 8.0; 0.1 mM EDTA) or Buffer EB (10 mM Tris-HCl, pH 8.5) to bring the volume up to 50 µL.

2. For each sample, make the following End Repair Master Mix:

End Repair Master Mix	
Component	Volume per reaction (µL)
End Repair Buffer	6
End Repair Enzyme	3
Total volume:	9

   Tip If there is precipitate in the End Repair Buffer, vortex until the precipitate becomes clear in solution. The resulting Master Mix is viscous and requires careful pipetting.

3. Pulse-vortex the master mix for 10 sec, then briefly centrifuge. Keep the master mix on ice.
4. Add 9 µL of End Repair Master Mix to each well. Using a pipette set to 40 µL, pipette 10 times to mix, then seal the plate. Alternatively, seal the plate and vortex mix for minimum of 10 seconds.
5. Run the following thermal cycler program:

End Repair program		
Step	Temperature* (°C)	Time
End repair	20	30 min
Hold	4	Hold

   * Set the lid temperature to OFF, or to 40°C.

   While the end repair program runs, make the Ligation 1 Master Mix in preparation for the Perform post end repair cleanup steps.

Ligation 1 Master Mix	
Component	Volume per reaction (µL)
Ligation 1 Buffer	25
Ligation 1 Adapter	2
Ligation 1 Enzyme	3
Total volume:	30

6. Pulse vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice until ready to use.

   Important: Use extra caution when handling Ligation 1 and Ligation 2 adapter tubes: never handle Ligation 2 adapter before or during Ligation 1 Master Mix setup and handling. Trace contamination of Ligation 2 adapter into Ligation 1 adapter has been shown to induce adapter-dimer formation.

7. After the End Repair program reaches 4°C, proceed immediately to Perform post-end repair cleanup.

End repair cleanup

Note: Before starting cleanup, make sure the Ligation 1 Master Mix has already been prepared.

1. Thoroughly resuspend AMPure XP beads before use, then add 147.5 µL of AMPure beads (2.5X volume) to each well and pipette 10 times to thoroughly mix.
2. Incubate the plate at room temperature for 10 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If the solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.
4. With a clean P20 pipette tip, remove and discard any trace amount of supernatant that remains.

   Important: Be careful not to remove any beads.
5. Keeping the plate on the magnet, add 160 µL of 80% ethanol and incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Use a P20 pipette tip to remove any residual ethanol.
8. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.
9. Proceed immediately to Ligation 1.

Ligation 1

1. Remove the plate from the magnet, then add 30 µL Ligation 1 Master Mix.
2. Pipette mix a minimum of 10 times, then seal the plate.

   Important: Make sure the samples are thoroughly mixed and that the beads are fully resuspended before proceeding.
3. Run the following thermal cycler program:

Ligation 1 program		
Step	Temperature* (°C)	Time
Ligation	20	15 min
Inactivation	65	15 min
Hold	4	Hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set the thermal cycler heat block to 105°C.

   Safe Stop: The plate can temporarily remain at 4°C (no more than 2 hours). It is normal for beads to settle during this reaction.
4. Proceed to Ligation 2.

Ligation 2

1. For each sample, prepare the Ligation 2 Master Mix.

Ligation 2 Master Mix	
Component	Volume per reaction (µL)
Ligation 2 Buffer	4.5
Ligation 2 Adapter	4
Ligation 2 Enzyme A	0.5
Ligation 2 Enzyme B	1
Total volume:	10

2. Pulse-vortex the master mix for 10 seconds, then briefly centrifuge. Keep the master mix on ice until ready to use.
3. Add 10 µL of the Ligation 2 Master Mix to each well.
4. Using a pipette set to 35 µL, pipette 10 times to mix, then seal the plate.

   Important: Ensure the samples are thoroughly mixed, and make sure the beads are fully resuspended before proceeding.

   Note: If necessary, briefly centrifuge to collect contents to the bottom of the wells.

5. Run the following thermal cycler program:

Ligation 2 program		
Step	Temperature* (°C)	Time
Ligation	65	30 min
Hold	4	Hold

*If possible, set lid temperature to 70°C. If lid cannot be programmed, set the thermal cycler heat block to 105°C.

6. After thermal cycler program is completed, proceed immediately to Perform post-ligation 2 cleanup.

Ligation 2 cleanup

1. Add 100 µL of PEG/NaCl (2.5X volume) to each well, then pipette 10 times to mix.
2. Incubate the plate at room temperature for 10 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.

4. Use a clean P20 pipette tip to remove and discard any trace amount of supernatant that remains.

   Important: Be careful not to remove any beads.

5. Keeping the plate on the magnet, add 160 µL of 80% ethanol and incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Use a P20 pipette tip to remove any residual ethanol.

   Important: Make sure all the ethanol has been removed before proceeding.

8. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.

9. Remove the plate from the magnet, then add 20 µL of Buffer EB.
10. Seal the plate, then gently vortex (use 70% vortex capacity) to resuspend beads.
11. Allow the plate to incubate at room temperature for 5 minutes to elute DNA off the beads.
12. Place the plate on a magnet and wait for the beads to be cleared from the liquid (approximately 1–2 minutes).

    Note: Depending on the strength of your magnet, you may need to wait longer.

13. Carefully transfer 20 µL of the cleared liquid containing the eluted DNA into a new well. Proceed to Perform PCR amplification or pause here.

    Safe Stop: The plate can be stored at –20°C overnight.

PCR amplification using Singular Index Primers

Note: To create NGS libraries compatible with the G4 Platform, use Singular Genomics specific index primers. Sample index barcodes are introduced during PCR; double check that a unique primer pair is used for each sample.

1. Add 5 µL of Singular Dual Index PCR Primers to each well.
2. Add 25 µL of xGen 2x HiFi PCR Mix to each well, then pipette 10 times to mix.
3. Seal the plate, then briefly centrifuge.
4. Run the following thermal cycler program:

   Note: Only use standard cycling conditions for the PCR program. Fast cycling has been shown to negatively impact library yields.

PCR program			
Step	Temperature* (°C)	Time	Cycles
Polymerase activation	98	45 sec	Varies based on sample input (see Table 1)
Denature, Anneal, Extend	98	15 sec	
	60	30 sec	
	72	30 sec	
Final Extension	72	1 min	
Hold	4	Hold	

*Set lid temperature to 105°C. If the lid cannot be programmed, set the thermal cycler block to 105°C.

Table 1. Recommended PCR cycling parameters to yield >500 ng.

Number of cycles		
Input mass (ng)	gDNA or dsDNA	FFPE*
1	11–13	14–16
10	9–11	11–13
25	7–9	9–11
100	5–7	7–9
250	4–6	6–8

* For lower-quality FFPE samples, we recommend using the higher number of recommended PCR cycles from Table 1. For very low quality FFPE (DIN 1- 2), you may not obtain 500 ng, but we do not recommend increasing the number of PCR cycles.

5. After the program completes, proceed to post-PCR cleanup.

PCR cleanup

1. Add 65 µL of AMPure beads (1.3X volume), or similar bead-based DNA purification product, to each well, then pipette 10 times to thoroughly mix.
2. Incubate the plate at room temperature for 5 minutes.
3. Place the plate on a magnet and wait for the liquid to clear completely or at least for 2 minutes.

   Important: If solution is not clear after 2 minutes, keep the plate on the magnet until solution clears. If beads are discarded, sequencing library yield and quality may suffer.
4. Remove and discard the cleared supernatant; make sure not to remove any beads.

   Important: Be careful not to remove any beads.
5. Keeping the plate on the magnet, add 160 µL of 80% ethanol, then incubate for 30 seconds.
6. Remove and discard the supernatant.
7. Repeat the wash step for a total of two washes.
8. Use a P20 pipette tip to remove any residual ethanol.

   Important: Make sure all the ethanol has been removed before proceeding.
9. Dry the beads at room temperature for 1–3 minutes.

   Important: Never allow the beads to dry for >5 minutes; over-dry beads will decrease final library yield.
10. Remove the plate from magnet, then add 31 µL of Buffer EB.
11. Seal the plate and gently vortex (use 70% vortex capacity) to resuspend beads.
12. Allow the plate to incubate at room temperature for 5 minutes to elute DNA off beads. Then place the plate on a magnet and wait for the liquid to clear completely for 1–2 minutes.

    Note: Depending on the strength of your magnet, you may need to wait longer.
13. Carefully transfer 30 µL of eluted DNA into a new well.

The library is now ready for quantification and quality check. Information on pre-sequencing QC can be found on Singular Genomics website (https://singulargenomics.com/g4/support). If performing hybridization capture, see Appendix A.

Library performance

Example libraries were prepared from a 1 ng input of cfDNA reference material (Horizon) using the xGen cfDNA & FFPE DNA Library Prep MC v2 Kit with Singular Dual Index PCR Primers. After library preparation, hybridization capture was performed as a three-plex using a xGen Custom Hyb Capture panel with the xGen Hybridization and Wash v2 Kit with custom blocking oligos and amplification oligos designed for use with libraries containing Singular-specific index primers. In Figure 1, a Tapestation library trace (Agilent) shows a hybridization captured library. Table 2 shows the sequencing metrics obtained from sequencing 15 hybridization captured libraries on the G4 Sequencing Platform.

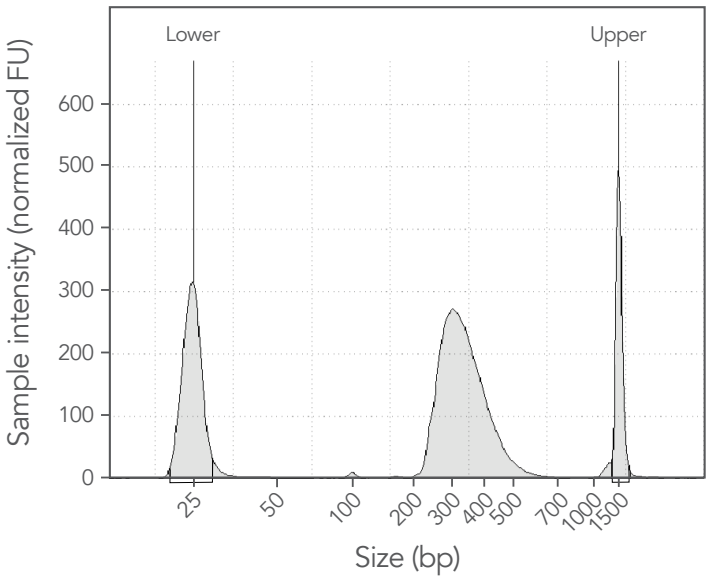

Figure 1. Representative TapeStation trace for a hybridization captured xGen cfDNA & FFPE DNA Library Prep Kit compatible with G4 Platform. The resulting library showed a peak height of ~300bp; this is the expected library size after adding the adapters and index sequences to the cfDNA sample.

Table 2 shows the sequencing metrics obtained on the G4 Sequencing Platform. Two individual flow cells were ran on the same sequencing run. The sequencing run contained 15 hybridization captured libraries prepared using the xGen cfDNA & FFPE Library Prep Kit. Both flow cells passed all instrument quality specifications.

Table 2. Sequencing metrics obtained on the G4 Sequencing Platform.

Flow Cell	Read pairs	R1 Percent of Called Bases >= Q30	R2 Percent of Called Bases >= Q30	% Non-Demuxed Reads	R1 Accuracy*	R2 Accuracy*
1	238 M	92.97	94.25	1.1	99.89	99.91
2	267 M	93.77	94.91	1.1	99.90	99.92

Appendix A: Hybridization capture

To perform hybridization capture on the libraries, follow the **xGen hybridization capture of DNA libraries protocol** with the following adjustments. Because the libraries contain Singular specific sequences, custom blocking oligos and amplification primers are necessary. Do not use the xGen Universal blockers or the xGen Library Amplification mix as the sequences are not compatible with the libraries generated with the Singular index primers. **Contact us** to order the custom blockers and amplification primers. No other adjustments are needed to the hybridization protocol.

Appendix B: Fixed UMI sequences

The Ligation 1 Adapter contains 32 optimized, fixed UMI sequences that are 8 base pairs in length.

Adapter name	UMI sequence	Adapter name	UMI sequence
UMI_1	GAGACGAT	UMI_17	GCACAACT
UMI_2	TTCCAAGG	UMI_18	GCGTCATT
UMI_3	CGCATGAT	UMI_19	GAAGGAAG
UMI_4	ACGGAACA	UMI_20	ACTGAGGT
UMI_5	CGGCTAAT	UMI_21	TGAAGACG
UMI_6	GCTATCCT	UMI_22	GTTACGCA
UMI_7	TGGACTCT	UMI_23	AGCGTGTT
UMI_8	ATCCAGAG	UMI_24	GATCGAGT
UMI_9	CTTAGGAC	UMI_25	TTGCGAAG
UMI_10	GTGCCATA	UMI_26	CTGTTGAC
UMI_ 11	TCGCTGTT	UMI_27	GATGTGTG
UMI_12	TTCGTTGG	UMI_28	ACGTTCAG
UMI_13	AAGCACTG	UMI_29	TTGCAGAC
UMI_14	GTCGAAGA	UMI_30	CAATGTGG
UMI_ 15	ACCACGAT	UMI_31	ACGACTTG
UMI_16	GATTACCG	UMI_32	ACTAGGAG

For analysis guidelines for processing sequencing data with UMIs see **this document**.

Revision history

Version	Release date	Description of changes
1	January 2023	Initital release.

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For more information, go to: **www.idtdna.com/ContactUs**

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